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Effects of cadmium on mitochondrial structure and function in different organs: studies on the soil centipede *Lithobius forficatus* (Myriapoda, Chilopoda)

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Abstract
Mitochondria are organelles that play a crucial role in cell physiology, cell death, and aging. They are among the first responders to different stressors that originate from the environment. Cadmium as a heavy metal affects different levels of body organization: from organs through tissues and cells to organelles. Based on our previous research results, we decided to check how the exposure to cadmium affects the functioning of mitochondria in different organs of soil living centipede *Lithobius forficatus*. The activity of mitochondria in somatic and germ cells has been analyzed using transmission electron microscopy (TEM), confocal microscopy, and flow cytometry. Changes in the mitochondrial membrane potential and mitochondrial dismutase (MnSOD) activity in relation to the accumulation of reactive oxygen species (ROS) caused by cadmium exposure have been studied. Individuals were divided into 3 experimental groups depending on cadmium concentration in soil. Changes in mitochondrial ultrastructure caused by cadmium are tissue-dependent and associated with an increase of ROS levels. The system of ROS and MnSOD activation works more efficiently in the case of gonads than in the digestive system. While the short-term cadmium exposure alters the fine structure of both the somatic and germ-line cells in gonads, the long-term cadmium exposure causes mitochondrial ultrastructure regeneration.

Keywords: *Mitochondria, JC1, mitochondrial membrane potential, somatic cells, germ cells*

1. Introduction

Heavy metals such as cadmium can enter the freshwater, marine or terrestrial environment due to anthropogenic activity or natural geological processes. Cadmium is a metal which, due to its high concentrations in air, water, and soil, rapid transport in the soil-plant-animal trophic chain, and high toxicity, is considered to be one of the most dangerous to both aquatic and terrestrial organisms (Descamps et al. 1996; Satarug et al. 2003; Wang et al. 2013; Stalmach et al. 2015; Tarnawska et al. 2019). It affects different levels of body organization: from organs through tissues and cells to organelles and all the compounds synthesized (Vega et al. 1989; Siekierska & Urbańska-Jasik 2002; Siekierska 2003; Hödl et al. 2010; Babczyńska et al. 2011; Takacs et al. 2016; Yuan et al. 2016; Bednarska et al. 2016, 2019; Augustyniak et al. 2017). Mitochondria are one of the organelles considered to be intracellular targets of cadmium. Thus mitochondrial dysfunction may be due to this metal's toxicity (Early et al. 1992; Miccadei

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& Floridi 1993; Wallace & Starkov 2000; Tang & Shaikh 2001; Sanni et al. 2008; Cannino et al. 2009; Hödl et al. 2010). Apart from being the main source of ATP, NADH, and GTP, they participate in the biosynthesis of amino acids, phospholipids, and reactive oxygen species (ROS), calcium signaling, oxidative stress, intermediary metabolism, programmed cell death, e.g., apoptosis, and aging (Detmer & Chan 2007; Hödl et al. 2010; Picard et al. 2013; Bratic & Larsson 2013; Kühlbrandt 2015; Włodarczyk et al. 2019). Mitochondria interact with other organelles (e.g., the endoplasmic reticulum, nuclei) and membranes, coordinating their cellular biological functions. When the crosstalk between mitochondria and other organelles fails, and the mitochondria are dysfunctional, cell homeostasis is disrupted. Hence, mitochondrial damage can lead to irreversible cell transformations (Nunnari & Suomalainen 2012; Xia et al. 2019). Because they play a crucial role in cell physiology (e.g., phosphorylation of ADP, signaling through mitochondrial ROS, calcium signaling, etc.), cell death, and aging, mitochondria are among the first responders to different stressors, including heavy metals (e.g., cadmium) (Hödl et al. 2010; Bednarska & Świątek 2016).

Previous studies have revealed the ultrastructural changes in mitochondria as well as the changes in the mitochondrial enzyme systems caused by cadmium (Early et al. 1992; Tang & Shaikh 2001; Cannino et al. 2009; Hödl et al. 2010; Wang et al. 2013). As it has been shown, the damage of the mitochondrial enzyme system by the accumulation of ROS (superoxide, hydrogen peroxide, hydroxyl radical) causes changes in the mitochondrial membrane potential (Koizumi et al. 1994; Cannino et al. 2009) and activation of antioxidant enzymes, e.g., dismutases (SOD) (Yao et al. 2007; Ramalho-Santos et al. 2009; Włodarczyk et al. 2019). Four classes of SOD enzymes have been distinguished depending on the metal cofactors: Manganese Superoxide dismutase/ Mitochondrial Superoxide dismutase (MnSOD), Copper, Zinc Superoxide dismutase (Cu/ZnSOD), Iron Superoxide dismutase (FeSOD), and Nickel Superoxide dismutase (NiSOD) (Zelko et al. 2002). Numerous intracellular and extracellular factors can induce MnSOD expression. Hence it is treated as an essential antioxidant enzyme that plays a crucial role in protecting cells against oxidative stress (Holley et al. 2010; Sarsour et al. 2012; Candas & Li 2014). To better understand the impact of cadmium on animals and the processes of cell protection, we decided to analyze mitochondrial alterations caused by short- and long-term cadmium exposure in different organs. Although cadmium is considered as a mitochondria-damaging metal, little is still known how it affects the mitochondria of soil organisms. We chose the well-known and widespread European centipede *Lithobius forficatus* (Myriapoda, Chilopoda, Lithobiomorpha). Its body has a simple structure and it is an omnivorous species: it is a predator but also feeds on the litter with organic and inorganic matter. Due to the fact that this species plays an important role in soil ecosystems, it seems to be an interesting species in ecotoxicological studies (Eisenbeis & Wichard 1987; Ostrowska et al. 1991; Lewis 2009). Our previous studies on *L. forficatus* revealed different changes and mechanisms activated after short- and long-term cadmium exposure. Comparing different organs, i.e. the midgut, salivary glands and the fat body at the ultrastructural level, we found that they can react differently to the same stressor, as well as to the same concentration and time of exposure. Eventually, the crosstalk between different cell death types occurs, which could be caused by different mechanisms (Rost-Roszkowska et al. 2020a, 2020b). Therefore, here we wanted to investigate the precise relationship between the general changes of some organs' structure and modifications connected with mitochondrial functioning. Due to the lack of data on the effect of heavy metals on various organs, the aim of these studies was to compare mitochondrial changes in somatic cells of different organs (salivary glands, midgut epithelial cells, somatic cells of gonads) and germ-line cells (e.g., oocytes, spermatocytes). The activity of mitochondria has been estimated by detecting the presence of mitochondrial superoxide dismutase (MnSOD), changes in the mitochondrial membrane potential, and their ultrastructure. It enabled us to assess the time-dependent and tissue-dependent differences caused by cadmium exposure.

2. Material and methods

2.1. Material

L. forficatus adults were harvested from May to October from non-polluted yards and forests of southern or central Poland (e.g., Żywiec, Poznań) and adapted to laboratory conditions for several weeks as described by Chajec et al. (2012) and Rost-Roszkowska et al. (2020a, 2020b).

2.2. Experiment

Animals were cultured in 30-liter terraria filled with the peat-based soil (room temperature, RT) with the properties described in our previous paper (Rost-Roszkowska et al. 2020a). Animals that were bred in cadmium-free soil were the control group in the experiment (**C group**). They were fed *ad libitum* with forest litter, detritus, and *Chironomus* larvae. The remaining animals were bred in soil with the addition of cadmium in the form of $CdCl_2$.

Hence, the animals were divided into experimental groups (Figure 1) according to our previous studies (Rost-Roszkowska et al. 2020a, 2020b) and literature data (Descamps et al. 1996; Vandenbulcke et al. 1998a, 1998b): **Cd1 –** animals bred in a soil containing 80 mg Cd kg^{-1} for 12 days (short-term exposure); **Cd2 –** animals bred in a soil containing 80 mg Cd kg^{-1} for 45 days (long–term exposure) (Table I). All the specimens were fed *ad libitum* as animals from the control group.

The animals were anesthetized with chloroform, and four organs were isolated from their body – the midgut, salivary glands, ovaries, and testis. The organs were prepared for the analysis using histological, histochemical, and immunohistochemical methods described in Table I.

2.3. Methods

2.3.1. Light and transmission electron microscopy (TEM). Isolated organs (midgut, salivary glands, ovaries, and testis) after fixation with 2.5% glutaraldehyde and 2% osmium tetroxide (4°C, 1.5 h each) were prepared (dehydrated, cut, and stained) according to standard methods used for TEM (Chajec et al. 2012; Rost-Roszkowska et al. 2020a, 2020b) Semi-thin (0.8 µm thick) sections were observed using an Olympus BX60 light microscope, while ultra-thin (70 nm) sections were examined using a Hitachi H500 transmission electron microscope.

2.3.2. Confocal microscopy. ***JC1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl-carbocyanine iodide) staining***. JC-1 is commonly used to differentiate cells with polarized mitochondria (orange fluorescence) and a depolarized mitochondria (green fluorescence) (Salvioli et al. 1997). The isolated organs (Table I) were stained with JC-1 dye and DAPI, according to Włodarczyk et al. (2017) and visualized using an Olympus FluoView FV1000 confocal microscope.

Dihydroethidium (DHE) – evaluation of reactive oxygen species (ROS) production. Isolated salivary glands, midgut, testis, and ovaries

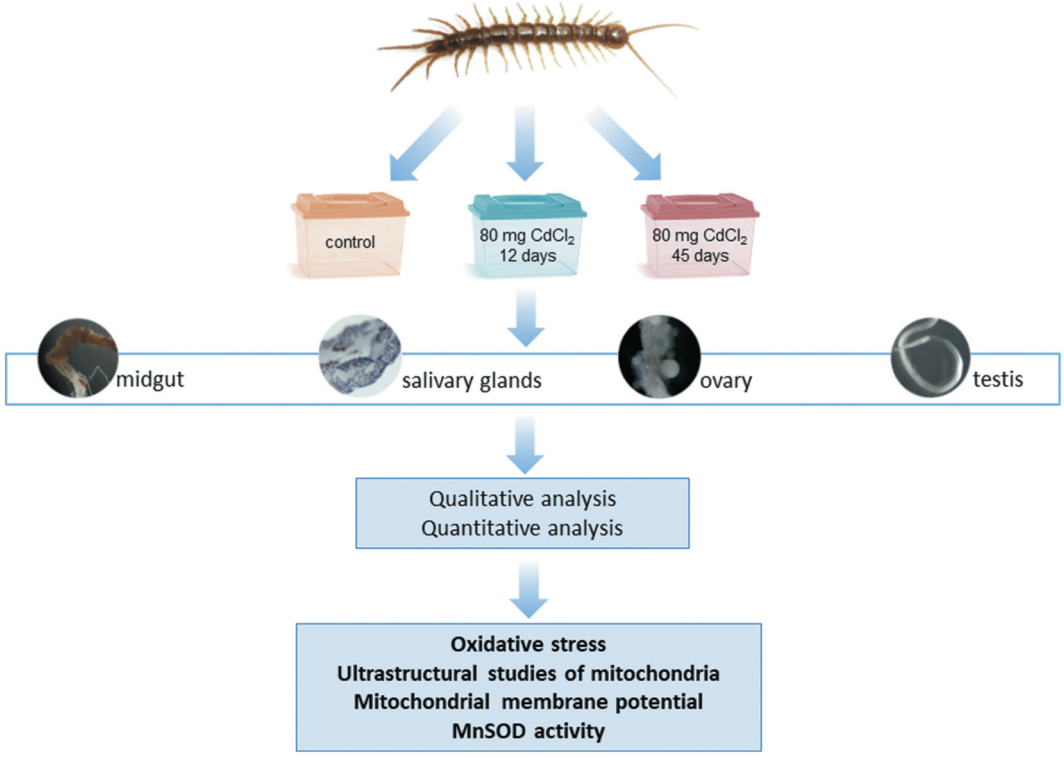

Figure 1. The scheme of the experiment described in chapter 2.

Table I. Number of specimens of each sex of *L. forficatus* examined.

Experimental group	Confocal microscopy		Flow cytometry		Fluorescent microscopy	Western blot
	JC1	DHE	JC1	DHE	MnSOD	MnSOD
C	2	2	5–6	5–6	5	5
Cd1	2	2	5–6	5–6	5	5
Cd2	2	2	5–6	5–6	5	5

were labeled with DHE and DAPI, as was precisely described by Włodarczyk et al. (2019) and examined with an Olympus FluoView FV1000 confocal microscope.

2.3.3. Fluorescent microscopy. ***Superoxide dismutase (SOD) detection.*** The isolated organs (midgut, salivary glands, ovaries, testis) without fixation were embedded in a tissue-freezing medium (Jung) and cryocut (5 μm thick sections). Then, the slides were blocked with BSA and incubated with the primary antibody: anti-MnSOD rabbit polyclonal antibody (1:500; Stressgen), goat anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (1:1000, Invitrogen) and DAPI according to protocols described by Włodarczyk et al. (2019) and eventually analyzed with an Olympus BX-60 fluorescence microscope.

2.3.4. Western blot analysis. Individuals of *L. forficatus* from the control group were anesthetized, midguts were dissected (5 per sample), and eventually, total protein concentration was measured according to the Bradford method (1976) and previously described in our paper (Włodarczyk et al. 2019). The detection of superoxide dismutase (MnSOD) was performed. The Western blot analysis was performed according to protocol described in our previous study (Włodarczyk et al. 2019).

2.3.5. Flow cytometry. The four organs isolated from each experimental group's specimens were mechanically fragmented with scissors and homogenized for obtaining the cell suspension (Włodarczyk et al. 2019). In the case of gonads, these organs were examined without distinguishing between somatic and germ cells because flow cytometry does not differentiate cell types.

Muse Oxidative Stress Kit (Merck Millipore, № MCH100111) was used for the quantitative analysis of cell populations undergoing oxidative stress - ROS negative (live cells) and ROS positive cells (cells exhibiting ROS) were distinguished (Włodarczyk et al. 2019). The measurements were performed using the Muse Cell Analyzer (Millipore).

JC-1 – quantitative assessment of cells with depolarized mitochondria. The cell suspension was incubated with JC-1 solution according to Włodarczyk et al. (2017) and analyzed using flow cytometry (Beckman Coulter Instrument FC 500) with a 488 nm argon laser using the MXP software Beckman Coulter program. The results were described as the percentage of cells with depolarized mitochondria.

2.3.6. Statistical analyses. Statistical elaboration (the Shapiro–Wilk test, Levene's test of equality of error variances, the Tukey test; N = 5–6, $p < 0.05$) was accomplished with STATISTICA 13 (software package system, version 13.0, http://www.statsoft.com) (Wilczek et al. 2019).

3. Results

3.1. Populations of cells undergoing oxidative stress

The use of dihydroethidium (DHE) for *L. forficatus* salivary glands, midgut, ovaries, and testis has shown a diverse distribution of ROS in all experimental groups. Weak signals originated from some of the cells in all organs in the control group. The signals from midgut cells were stronger than in the remaining organs in all experimental groups. The signals emitted by cells of salivary glands, midgut, and ovaries were stronger in Cd1 and Cd2 experimental groups than in the animals from C group. However, the signals that originated from the testis of animals treated with cadmium for 12 days were the strongest in comparison to C and Cd2 groups (Figure 2(a-l)).

The quantitative analysis revealed that prolonging exposure to cadmium increased the percentage of ROS+ cells in analyzed organs, compared to the control group. Independently of organ, the highest number of ROS+ cells was recorded in individuals from the Cd2 group (salivary glands – 26.9 ± 2.0; midgut – 45.3 ± 2.1; ovaries – 33.3 ± 1.9; testis – 29.2 ± 3.4). The ROS+ percentage in the Cd2 group was almost twice as high as in the Cd1 group. Only in the testis were no statistically significant differences in the number of ROS+ and ROS- cells between the Cd1 and Cd2 groups (Figure 3).

3.2. Ultrastructure of mitochondria

Mitochondria in all organs of the control group have an electro-dense matrix and distinct mitochondrial

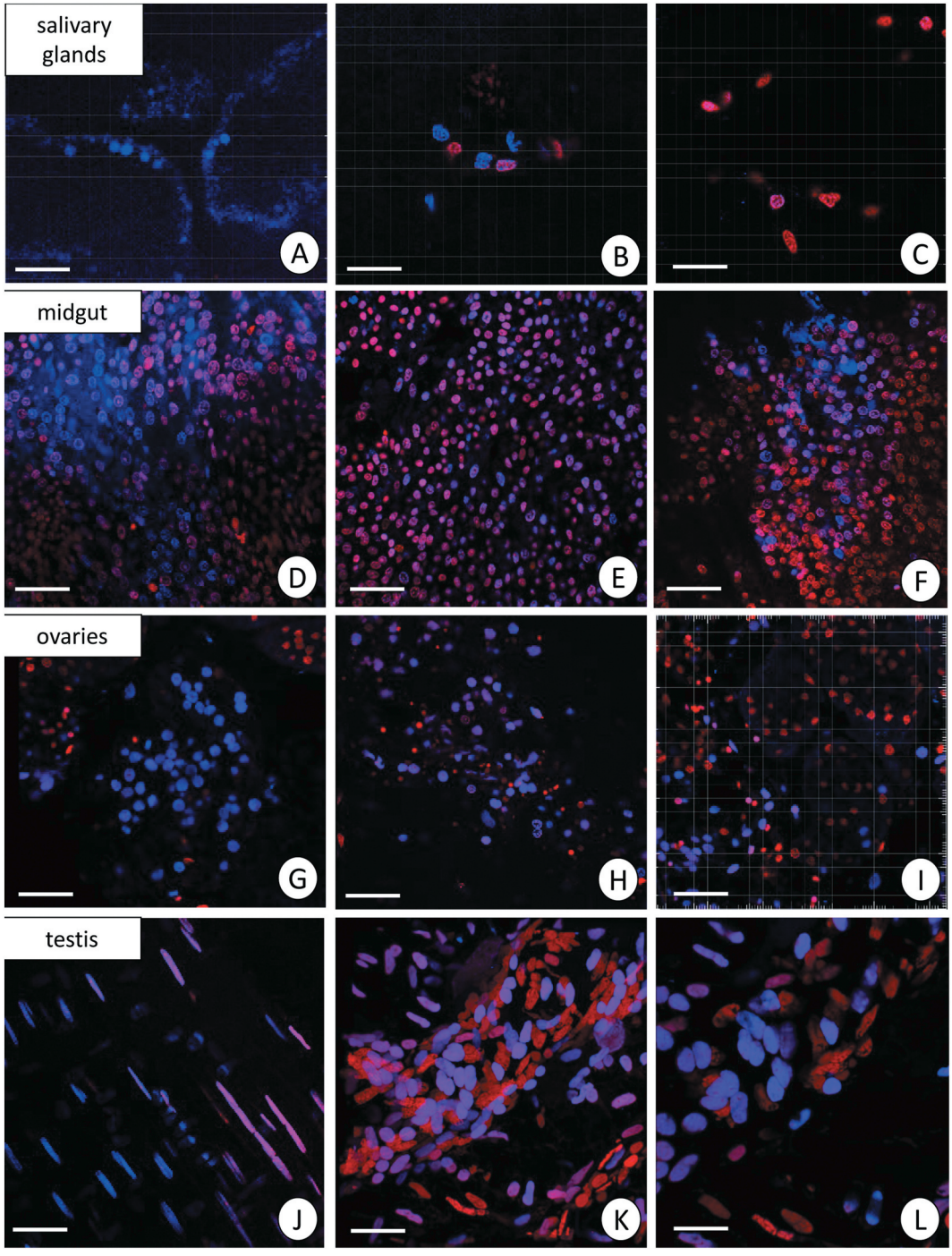

Figure 2. 3D representation of the DHE and DAPI staining of organs in *L. forficatus*. ROS-positive cells (red), nuclei (n, blue). Confocal microscope. (a) salivary glands in the C group. Scale bar = 16 μm. (b) salivary glands in the Cd1 group. Scale bar = 12 μm. (c) salivary glands in the Cd2 group. Scale bar = 12 μm. (d) midgut in the C group. Scale bar = 20 μm. (e) midgut in the Cd1 group. Scale bar = 20 μm. (f) midgut in the Cd2 group. Scale bar = 20 μm. (g) ovaries in the C group. Scale bar = 18 μm. (h) ovaries in the Cd1 group. Scale bar = 16 μm. (i) ovaries in the Cd2 group. Scale bar = 16 μm. (j) testis in the C group. Scale bar = 12 μm. (k) testis in the Cd1 group. Scale bar = 12 μm. (l) testis in the Cd2 group. Scale bar = 10 μm.

cristae. The ultrastructure of the mitochondria in salivary glands in both experimental groups Cd1 and Cd2, showed no alterations in comparison to the control group (Figure 4(a–c)). However, the most striking difference was the structural transformation of the mitochondria in the midgut digestive cells in the Cd1 group. The matrix of several mitochondria became electron-lucent, and the inner

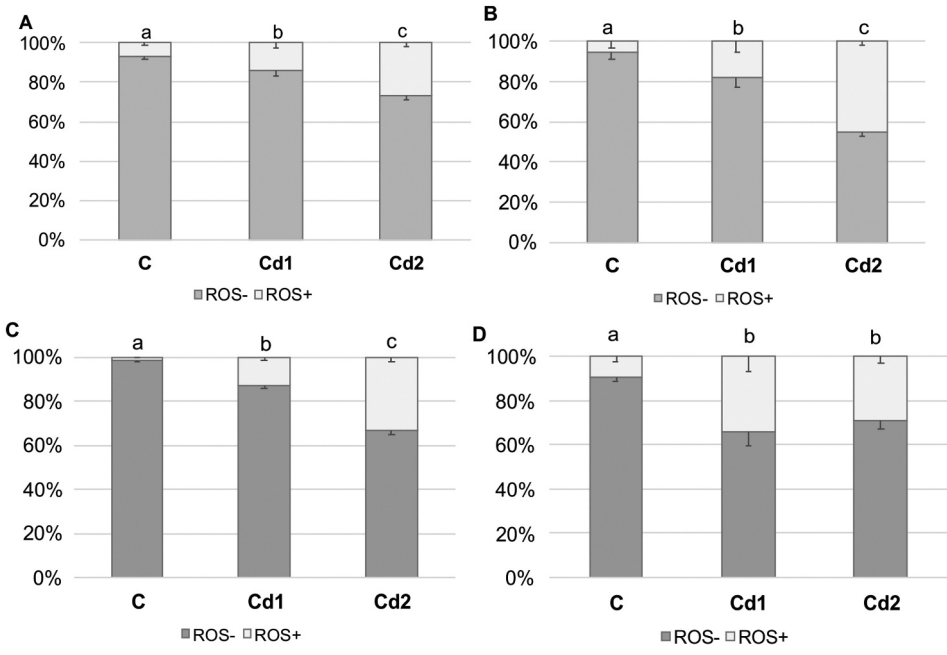

Figure 3. Percentage of ROS+ and ROS– cells (x± SD) in salivary glands (a), midgut (b), ovaries (c) and testis (d) of *L. forficatus* individuals from the control group (C) and exposed to metals (Cd1 and Cd2 groups). The different letters (a, b, c) indicate significant differences within each organ and parameter (Tukey test, $p < 0.05$; N = 5–6).

mitochondrial membranes are mainly smooth, and only some short cristae could be detected. However, numerous mitochondria exhibited structural alterations because they had vacuoles engulfing the nearby cytoplasm. The ultrastructure of mitochondria in animals originating from the Cd2 experimental group resembled that of the control group: normal-shaped mitochondria showed numerous cristae and an electron-opaque matrix (Figure 4(d–f)). No changes at the ultrastructural level were detected in the secretory or regenerative cells of the midgut epithelium. The transmission electron microscopy enabled us to distinguish two types of cells: the somatic and germ cells in both gonads (testis and ovaries); therefore, we could describe the differences between these two groups of cells. The ultrastructure of mitochondria in somatic cells of both gonads showed similar alterations to the midgut: changes appeared in mitochondria of the Cd1 experimental group. In contrast, in specimens from Cd2 experimental group, the mitochondria resembled those of the control animals (not shown). A similar relationship was detected after long-term cadmium exposure, and only in the germ cells of ovaries (e.g., oocytes) and testis (e.g., spermatocytes) several altered mitochondria were detected in the Cd1 experimental group (Figure 4(g–l)).

3.3. *Mitochondrial membrane potential*

The qualitative analysis using confocal microscopy revealed that the signals originating from active mitochondria with a high membrane potential (red signals) were strong in the midgut epithelium and salivary glands in specimens from the control group, but the midgut epithelium presented a more enormous amount of active mitochondria in comparison to salivary glands. In both organs, the signals originating from active mitochondria were weaker in Cd1 and Cd2 experimental groups. In contrast, numerous strong signals originating from inactive mitochondria with a low membrane potential (green signals) were detected in the control group (Figure 5(a–f)). In general, the midgut seems to be an organ with a much more considerable amount of active/non-active mitochondria. The confocal microscope enabled somatic and germ cells to be distinguished. We observed the differences in signals emitted by active and non-active mitochondria in animals from all experimental groups. Both cell types, the somatic and germ cells, in testis and ovaries showed a high number of active mitochondria (red signals), while sporadic green signals were emitted by the non-active mitochondria of germ cells (Figure 5(g,j)). After short-term cadmium treatment, the number of non-active mitochondria

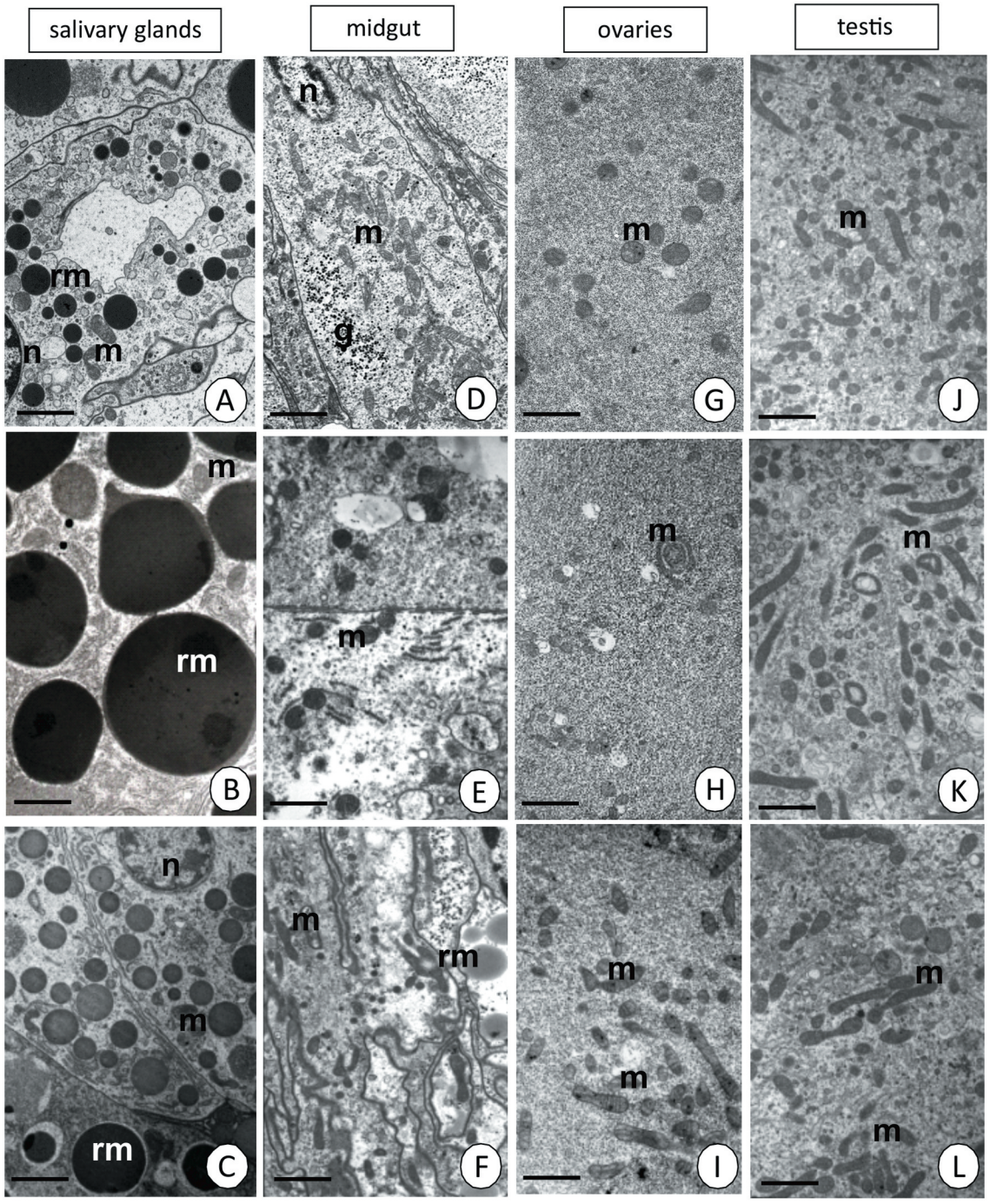

Figure 4. Transmission electron micrographs presenting the mitochondria in salivary glands (a–c), midgut (d–f), ovaries (g–i) and testis (j–l). Mitochondria (m), glycogen granules (g), nuclei (n), reserve material (rm). TEM. (a) C group. Scale bar = 2 μm. (b) Cd1 group. Scale bar = 1.2 μm. (c) Cd2 group. Scale bar = 2 μm. (d) C group. Scale bar = 2 μm. (e) Cd1 group. Scale bar = 1.2 μm. (f) Cd2 group. Scale bar = 2 μm. (g) C group. Scale bar = 1.1 μm. (h) Cd1 group. Scale bar = 1.6 μm. (i) Cd2 group. Scale bar = 1.3 μm. (j) C group. Scale bar = 1.5 μm. (k) Cd1 group. Scale bar = 1.4 μm. (l) Cd2 group. Scale bar = 1.1 μm.

strongly increased in both types of cells in ovaries, resulting in an increase of green signals emitted by non-active mitochondria. Long-term cadmium exposure led to an increased number of active mitochondria in the somatic cells of the ovary (red signals). In contrast, signals emitted by non-active

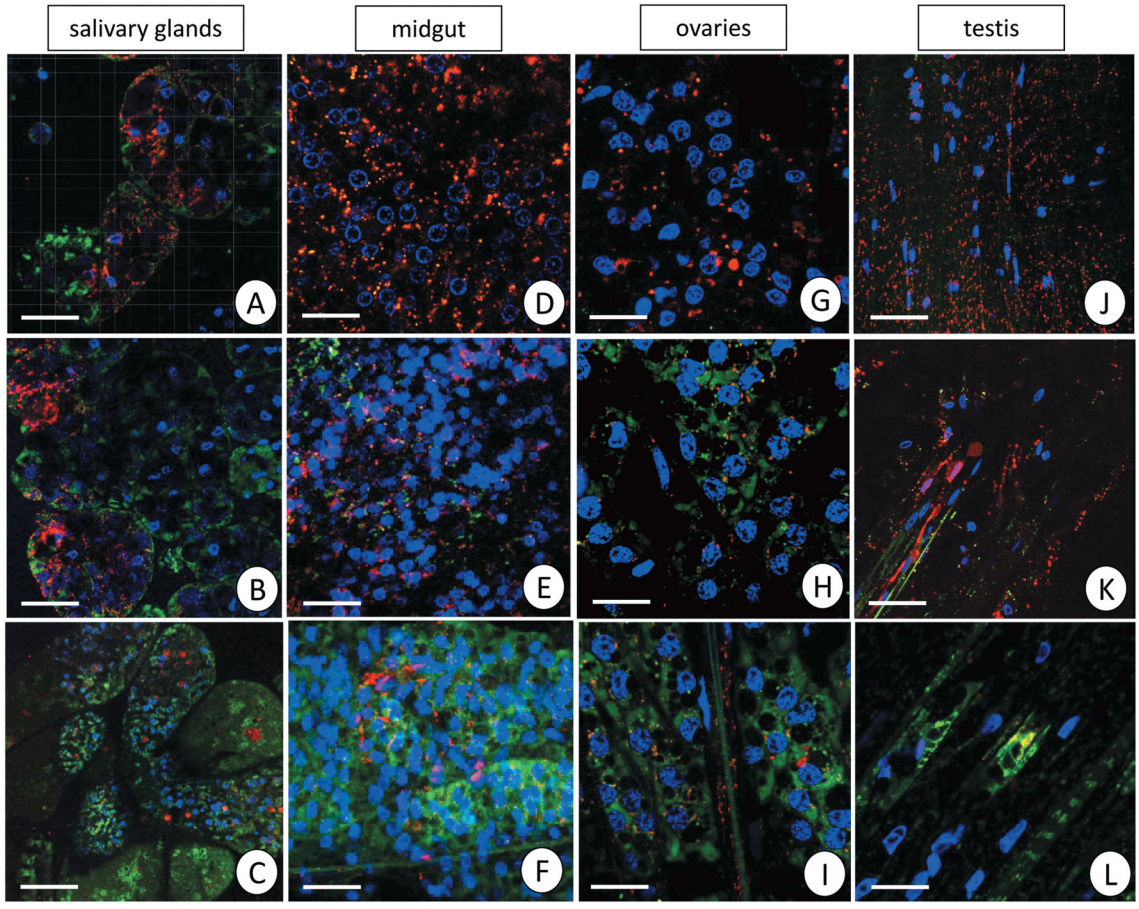

Figure 5. Mitochondrial potential in the *L. forficatus* organs. Active mitochondria with a high membrane potential (red signals), inactive mitochondria with a low membrane potential (green signals), nuclei (blue signals). JC–1 cationic dye, DAPI staining. Confocal microscope. (a) salivary glands in the C group. Scale bar = 16 μm. (b) salivary glands in the Cd1 group. Scale bar = 16 μm. (c) salivary glands in the Cd2 group. Scale bar = 20 μm. (d) midgut in the C group. Scale bar = 12 μm. (e) midgut in the Cd1 group. Scale bar = 16 μm. (f) midgut in the Cd1 group. Scale bar = 16 μm. (g) ovaries in the C group. Scale bar = 12 μm. (h) ovaries in the Cd1 group. Scale bar = 10 μm. (i) ovaries in the Cd2 group. Scale bar = 10 μm. (j) testis in the C group. Scale bar = 16 μm. (k) testis in the Cd1 group. Scale bar = 16 μm. (l) testis in the Cd2 group. Scale bar = 10 μm.

mitochondria were mainly detected in germ cells (Figure 5(h–i)). In the testis, the short-term cadmium exposure caused a slight increase in the number of non-active mitochondria, which emitted green signals in somatic cells. The germ cells still emitted green signals originating from non-active mitochondria. After 45 days of cadmium treatment, both the somatic and germ cells emitted only green signals originating from non-active mitochondria (Figure 5 (k–l)).

The quantitative analysis showed that independent of time exposure to cadmium, an increase in the number of cells with depolarized mitochondria was detected in analyzed organs compared to the control group (Figure 6(a–d)). An almost threefold increase in the number of cells with low mitochondrial transmembrane potential was detected in salivary glands ($P = 0.03$) (Figure 6(a)). In contrast, a fourfold increase in the ovary ($P = 0.04$) and a sevenfold increase in testis ($P = 0.0002$) were found (Figure 6(c,d)). In turn, 75% more cells with depolarized mitochondria were registered in the intestine of individuals exposed to cadmium for 45 days ($P = 0.03$; Cd2 group) (Figure 6(b)) than the control group. Independently of organ, there was no statistically significant difference in the number of cells with depolarized mitochondria between the Cd1 and Cd2 experimental groups.

3.4. *MnSOD activity*

The specificity of the antibodies was confirmed by the Western blot technique (Figure 7). The immunofluorescent method for detecting superoxide dismutase (MnSOD) at the light microscope level revealed a low level of this enzyme in the midgut

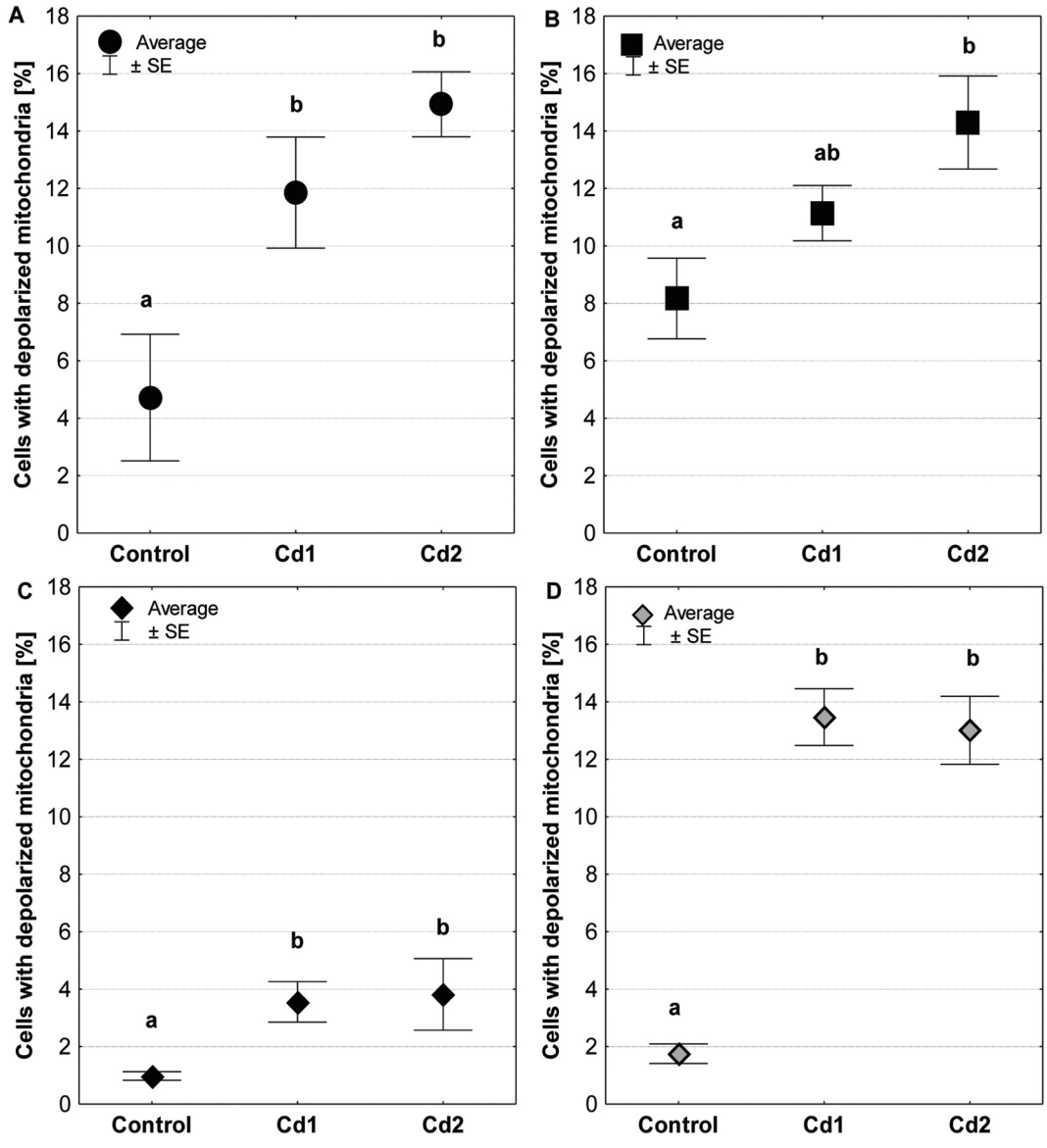

Figure 6. Percentage of cells with depolarized mitochondria (low ΔΨm) in salivary glands (a), midgut (b), ovaries (c) and testis (d) of individuals from the control group and exposed to metals (Cd1 and Cd 2 groups). The different letters (a, b) significance differences within each organ (Tukey test, $p < 0.05$; N = 4–7).

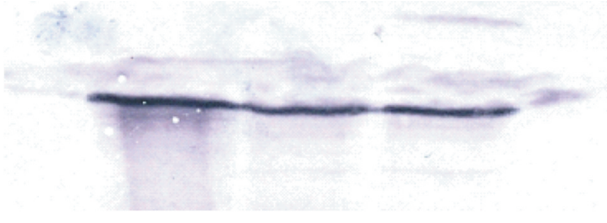

Figure 7. Western blot analysis of Superoxide dismutase (MnSOD) in the *L. forficatus* intestine (25 μg of protein per each line).

epithelial cells and salivary glands in the control group of *L. forficatus*. Short-term cadmium exposure activates MnSOD expression, but a more enormous amount of MnSOD was detected after long-term Cd exposure (Figure 8(a–f)). However, in the case of gonads, we observed that germ cells emitted more signals originating from MnSOD localization in comparison to somatic cells. These signals were the strongest in ovaries and testis of animals from the Cd2 experimental group (Figure 8(g–l)).

4. Discussion

Free radicals are derived from enzymatic and nonenzymatic reactions that naturally occur in cells or

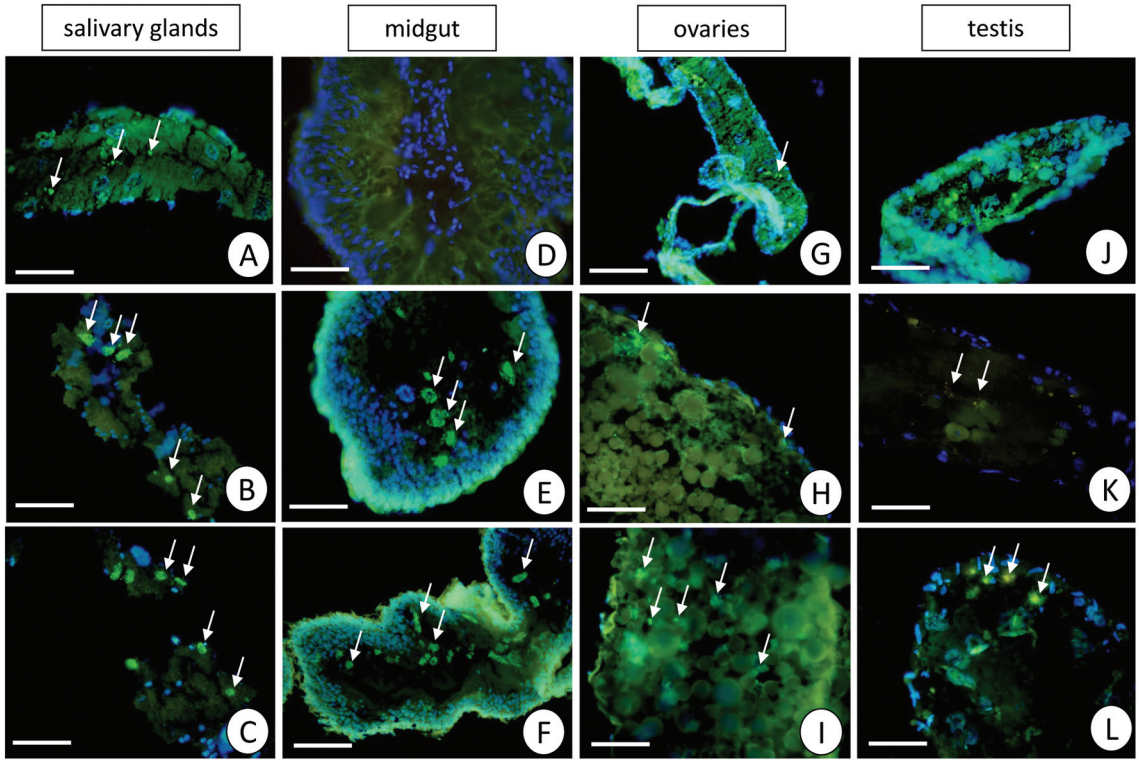

Figure 8. A fluorescent representation of the MnSOD localization (green) and DAPI staining in salivary glands, midgut, ovaries and testis of *L. forficatus*. Nuclei (blue). Fluorescent microscope. (a) salivary glands in the C group. Scale bar = 16 μm. (b) salivary glands in the Cd1 group. Scale bar = 16 μm. (c) salivary glands in the Cd2 group. Scale bar = 16 μm. (d) midgut in the C group. Scale bar = 24 μm. (e) midgut in the Cd1 group. Scale bar = 26 μm. (f) midgut in the Cd2 group. Scale bar = 26 μm. (g) ovaries in the C group. Scale bar = 26 μm. (h) ovaries in the Cd1 group. Scale bar = 16 μm. (i) ovaries in the Cd2 group. Scale bar = 16 μm. (j) testis in the C group. Scale bar = 24 μm. (k) testis in the Cd1 group. Scale bar = 20 μm. (l) testis in the Cd2 group. Scale bar = 18 μm.

can be caused by many environmental factors. They are involved in different physiological processes, that enable proper cellular functioning, including regulating of many intercellular signaling transduction or activation of the immune system. In the case of the reproductive system, they are involved in gonads' and germ cells' development and differentiation, as well as in fertilization. Their level determines cell functioning. When the ROS level is low, the cell remains in a quiescent state (Neckameyer & Matsuo 2008; Zhou et al. 2014; Lyublinskaya et al. 2015; Dziewięcka et al. 2017). The loss of homeostasis between ROS synthesis and their elimination activates the phenomenon called oxidative stress (Golden et al. 2002; Martindale & Holbrook 2002; Stalmach et al. 2015; Redza-Dutordoir & Averill-Bates 2016; Ahmad et al. 2017; Włodarczyk et al. 2019; Tarnawska et al. 2019). Animals have developed numerous defense mechanisms responsible for the neutralization of free radicals in cells and consequently, regulating the functioning of cells. This antioxidant defense system is composed of antioxidants, e.g., dismutases (e.g., MnSOD), catalase, glutathione, or cytochrome c released from mitochondria (Luo 2001; Babczyńska et al. 2011; Kaminskyy & Zhivotovsky 2014; Suganya et al. 2016; Augustyniak et al. 2016, 2017; Oropesa et al. 2017; Haque et al. 2018; Włodarczyk et al. 2019).

Toxic effects of cadmium are connected with its dose, period of exposure, the environment, and the age or developmental stage of animals (Neckameyer & Matsuo 2008; Liu et al. 2009; Babczyńska et al. 2011; Bednarska & Świątek 2016). Our research on the terrestrial centipede, which lives in soil polluted with high cadmium contamination, revealed that the presence of this heavy metal in the environment, not in the animal's food, can cause an increase in the level of ROS in somatic cells (here the salivary glands and midgut epithelium). The low level of ROS in analyzed organs in the control group related to natural physiological processes, while it gradually increased due to the prolongation of cadmium exposure. As it is known, the middle region of the digestive system – the midgut – is one of the barriers for the organism against external stressors (Leonard

et al. 2009; Janeh et al. 2019). The relationship between oxidative stress and MnSOD activation has been registered in the midgut epithelium in various invertebrates (Zhang et al. 2007; Yu et al. 2011; Suganya et al. 2016; Oropesa et al. 2017; Haque et al. 2018; Włodarczyk et al. 2019). The low level of MnSOD in the midgut epithelial cells and salivary glands in the control group of *L. forficatus* suggests that the level of ROS is probably connected with physiological processes. The increase in the number of signals emitted by MnSOD in these organs after short- and long-term cadmium exposure shows its activation as the response to a higher ROS level. However, while in the salivary glands and midgut epithelium our study confirms earlier data, the information connected with gonads and germ-line cells is rather poor in animals living in soil contaminated with toxic metals. Our study showed that while the ovaries presented the same phenomenon in the increase of the ROS level connected with periods of cadmium exposure, the testis emitted stronger signals of ROS accumulation after short-term cadmium exposure in comparison to long-term exposure. Up to now, similar studies have been presented for the female reproductive system, when ROS have been associated with its pathologies, because they were mainly detected in the somatic cells of ovaries (e.g., ovarian follicles) or reproductive ducts (e.g., the fallopian tube) (Agarwal et al. 2005). Higher than physiological levels of ROS activate the apoptosis of granulosa cells and cause the reduction of nutrient transfer to oocytes (Ahmad et al. 2017; Khazaei & Aghaz 2017). In testis, ROS are generated by mitochondria in germ-line cells whose cytoplasm is rich in these organelles because they constantly require ATP for motility and development (Griveau & Le Lannou 1997; Ahmad et al. 2017) but also for their ability to fertilize (Pelliccione et al. 2011; Sousa et al. 2011). When the ATP is required in male germ-line cells, the concentration of molecular oxygen in the mitochondria increases, leading to intensive activation of the antioxidant defense system (Said et al. 2005; Małota et al. 2019). We observed that in both gonads – testis and ovaries – of *L. forficatus* germ cells emitted more signals originating from MnSOD localization in comparison to somatic cells. These signals were the strongest in ovaries and testis of animals from the Cd2 experimental group, suggesting that MnSOD as a defense enzyme has been strongly synthesized in these cells compared to the somatic cells of gonads. In the terrestrial earthworm *Dendrobaena veneta*, the different ROS distribution in different types of cells in ovaries has been observed. The level of ROS was lower in somatic cells than in e.g. younger germ-line cells and post-vitellogenic oocytes. The level of MnSOD corresponds to ROS accumulation in this species. A high level of MnSOD activity has been noted in germ-line cells in comparison to somatic cells of the ovary (Faron et al. 2015). Generally, it is suggested that germ-line cells in ovaries of invertebrates should be more active metabolically than somatic cells (Tourmente et al. 1990; Świątek et al. 2001, 2004). However, in the testis, the number of germ cells is higher than in the ovaries, explaining the higher signals originating from MnSOD and ROS in the male gonads. The high level of ROS in the testis of the centipede from the Cd1 experimental group in comparison to the control and Cd2 groups probably is related to the fact that MnSOD is strongly involved in sperm protection causing the reduction of ROS in these cells after long-term cadmium exposure. This mechanism must occur much faster and more strongly than in the case of ovaries. We can conclude that in testis, the oxidative stress occurs after the short-term heavy metal exposure, while in ovaries, salivary glands, and midgut epithelium, it is activated after 12 days of cadmium exposure, but a longer period is needed for the proper functioning of antioxidative defense. In addition, this system works more efficiently in the case of gonads.

Mitochondrial dysfunction is the mechanism of cytotoxicity of cadmium because Cd affects the mitochondrial morphology (Miccadei & Floridi 1993; Wallace & Starkov 2000; Sokolova 2004; Hödl et al. 2010). Both the short- and long-term cadmium exposure of adult specimens of soil centipede did not cause any ultrastructural alterations of mitochondria in salivary glands, even though the number of non-active organelles with low mitochondrial potential increased after short- and long-term cadmium exposure. Measurements of the transmembrane mitochondrial potential ($\Delta\Psi m$) are considered as markers of all alterations that lead to cell death before any visible changes occur (Orrenius 2004; Zorova et al. 2018). The most striking differences were noted in the midgut epithelium in *L. forficatus* after short-term cadmium exposure. Numerous mitochondria showed signs of degeneration, and it was followed by an increase in the number of non-active mitochondria. It is suggested that mitochondrial dysfunction occurs according to inhibition or transformation of the respiratory complex rather than deletions of mitochondrial DNA (Siskova et al. 2010). The abnormalities in mitochondrial morphology, including abnormal cristae,

their vacuolization, and disruption of the mitochondrial membranes are connected with an increase of ROS levels, while the overexpression of MnSOD decreases their level together with the preservation of mitochondrial morphology (DeMartino et al. 1979; Faron et al. 2015). As we mentioned above, MnSOD in the Cd1 group was detected at a very low level. However, after long-term cadmium exposure (Cd2), the ultrastructure of these organelles in the midgut epithelium resembles that of the control group. It corresponds with a higher amount of MnSOD after long-term metal exposure and the higher ROS level. The increase in the number of mitochondria with low mitochondrial potential after long-term cadmium exposure suggests that their ultrastructure has been regenerated, although they are still not active. A similar process has been described in freshwater shrimp midgut epithelium (Włodarczyk et al. 2019).

Changes in both mitochondrial ultrastructure and mitochondrial potential could be associated with the activation of cell death (Kaminskyy & Zhivotovsky 2014; Redza-Dutordoir & Averill-Bates 2016; Sonakowska et al. 2016; Włodarczyk et al. 2017) because mitochondria play an essential role in the activation of cell death (Orrenius 2004). The inner-membrane structural alterations and morphological changes of mitochondria have been implicated in processes connected with programmed cell death and as a response to oxidative stress in different tissues and organs (Mannella 2008). Our previous studies on *L. forficatus* revealed that short-term Cd exposure activates autophagy in both the salivary glands and the midgut epithelium. In contrast, after the long-term metal exposure, this process is inhibited, while cell death (both necrosis and apoptosis) is involved in the response against the stressor (Rost-Roszkowska et al. 2020a, 2020b). The relationship between cadmium exposure and autophagy has been studied mainly in mammalian cells and tissues (Luo et al. 2016), but such a correlation has also been detected in invertebrates (Bednarska et al. 2016; Chiarelli et al. 2016; Wilczek et al. 2019). Comparing the present study with the previous one, we can state that when autophagy protects cells against cell death, the mitochondrial dismutase is not activated. Activation of cell death (here apoptosis and necrosis) corresponds to the intensive synthesis of MnSOD in both organs–the salivary glands and the midgut epithelium. The different response to the stressor suggests that the midgut epithelium is more sensitive to heavy metal presence than the salivary glands. Damage of mitochondria occurs differently in organs with different regenerative abilities (Dai et al. 2009; Velasquez-Vottelerd et al. 2015; Brandt et al. 2017). Additionally, it confirms the statement that the midgut epithelium as the middle region of the digestive system is considered one of the barriers in the organism against any stressors (Leonard et al. 2009). Eventually we could state that changes in mitochondrial ultrastructure caused by cadmium are tissue-dependent.

The quantitative analysis showed a distinct increase in the number of depolarized (non-active) mitochondria in ovaries and testis of *L. forficatus* after short- and long-term cadmium exposure. However, the confocal microscope enabled us to distinguish somatic and germ cells. The results showed that mostly red fluorescence originated from the active mitochondria in somatic cells, and green fluorescence was emitted by depolarized mitochondria in the germ-line cells of both gonads. The activity of mitochondria in somatic cells measured by red fluorescence has been recorded in the *Dendrobaena veneta* ovary. The germ-line cells in this species presented the lower oxidative activity of mitochondria (Faron et al. 2015). Similar results were obtained for *Xenopus laevis* during the formation of vitellogenic and post-vitellogenic oocytes (Mignotte et al. 1987; Kogo et al. 2011). It has been concluded that the level of mitochondrial activity is low in germ-line cells of animals (Tarazona et al. 2006; Kogo et al. 2011; Faron et al. 2015). In *Danio rerio*, the number of active and non-active mitochondria was equal in both types of cells (Zhang et al. 2008) or depending on the stage of oogenesis, the germ-line cells could be completely inactive (Tarazona et al. 2006; Kogo et al. 2011). The level of mitochondrial activity in the germ-line cysts during spermatogenesis in *D. veneta* was low, and the percentage of polarized mitochondria decreased in consecutive spermatogenesis stages (Małota et al. 2019). The somatic cells' main role in invertebrates gonads is the protection and support of germ-line cells, the synthesis and secretion of the material for the egg shells and hormonal regulation of oogenesis (Adiyodi & Adiyodi 1983a; Poprawa et al. 2002; Poprawa & Janelt 2019). Sometimes they participate in vitellogenesis (Telfer et al. 1982). In the testis, somatic cells take part in the separation of the developing group of germ cells, the nutritional support of the germ cells, the hormonal regulation of the spermatogenesis, and the secretion of the material for spermatophore (Adiyodi & Adiyodi 1983b; Minelli 2011). Therefore, the active state of mitochondria in somatic cells in both gonads of *L. forficatus* is connected with their functions.

The short-term cadmium treatment of soil centipedes caused an increase in the number of non-active mitochondria in somatic and germ-line cells in both gonads. However, the signals emitted were stronger in the case of ovaries than in testis. However, the changes were different after long-term cadmium exposure. In the testis, both the somatic and germ cells emitted only green signals originating from non-active mitochondria, while an increase in the number of active mitochondria in the somatic cells was detected in ovaries. Regardless of the transmission electron microscopy, the results suggest that in both somatic and germ-line cells, the alterations connected with e.g. vacuolization, only after the short-term cadmium exposure occurred. The long-term cadmium treatment did not alter the mitochondrial ultrastructure. Different periods of cadmium exposure can cause changes in respiratory activity followed by alterations in mitochondrial membrane potential (Cannino et al. 2008, 2009), deflecting mitochondrial membrane potential (Koizumi et al. 1994), and organelles bulging (Al-Nasser 2000). The early mitochondrial swelling and vacuolization seemed to be typical for Cd exposure, suggesting an obstruction of the oxidative metabolism (Ossola & Tomaro 1995). Because changes in mitochondrial ultrastructure and mitochondrial potential could be associated with activation of cell death (Orrenius 2004; Sonakowska et al. 2016; Włodarczyk et al. 2017), the analysis of cell death in gonads of *L. forficatus* exposed to cadmium is necessary. Additionally, the results connected with changes of mitochondria in gonads confirm our previous statement that mitochondria are still not active as after long-term Cd treatment, and their ultrastructure has been regenerated (Włodarczyk et al. 2019). Thus we suspect that additional mechanisms must be involved in processes related to mitochondrial activity, not only changes in their membrane potential and ultrastructural alterations.

5. Conclusions

Our research is the first in which changes in mitochondria between somatic cells of organs belonging to the digestive system, somatic cells in gonads and germ-line cells were compared according to cadmium presence in the environment of soil-dwelling organisms. We observed that: (1) abnormalities in mitochondrial morphology including abnormal cristae, their vacuolization and disruption of the mitochondrial membranes in all organs are connected with an increase of ROS levels; (2) changes in mitochondrial ultrastructure caused by cadmium are tissue-dependent; (3) oxidative stress strongly occurs after short-term metal exposure in testis, while in ovaries, salivary glands and midgut epithelium it is activated after short-term cadmium exposure, but a more extended period is needed for the proper functioning of antioxidative defense; (4) the system of ROS and MnSOD activation works more efficiently in the case of gonads than in the digestive system; (5) while the short-term cadmium exposure alters the ultrastructure of both the somatic and germ-line cells in ovaries and testis, the long-term Cd exposure causes the activation of mechanisms which enable the mitochondrial ultrastructure to regenerate; (6) the active state of mitochondria in somatic cells and non-active state of mitochondria in germ-line cells in both gonads in the non-treated animals are connected with their functions and are a common phenomenon. Additionally, comparing the results from this study and the previous one, we can conclude that when autophagy protects cells against cell death, the mitochondrial dismutase is not activated. Activation of cell death corresponds to the intensive synthesis of MnSOD in both the salivary glands and the midgut epithelium. An analysis of cell death processes in the gonads should be performed under experimental conditions to ensure this statement.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not concern any studies with human participants that were performed by any of the authors.

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